LETTER

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Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells

Kathryn Blaschke[1]*, Kevin T. Ebata[1]*, Mohammad M. Karimi[2,3], Jorge A. Zepeda-Martínez[4], Preeti Goyal[2], Sahasransu Mahapatra[4], Angela Tam[3], Diana J. Laird[1], Martin Hirst[3,5], Anjana Rao[4], Matthew C. Lorincz[2] & Miguel Ramalho-Santos[1]

DNA methylation is a heritable epigenetic modification involved in gene silencing, imprinting, and the suppression of retrotransposons[1]. Global DNA demethylation occurs in the early embryo and the germ line[2,3], and may be mediated by Tet (ten eleven translocation) enzymes[4–6], which convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC)[7]. Tet enzymes have been studied extensively in mouse embryonic stem (ES) cells[8–12], which are generally cultured in the absence of vitamin C, a potential cofactor for Fe(II) 2-oxoglutarate dioxygenase enzymes such as Tet enzymes. Here we report that addition of vitamin C to mouse ES cells promotes Tet activity, leading to a rapid and global increase in 5hmC. This is followed by DNA demethylation of many gene promoters and upregulation of demethylated germline genes. Tet1 binding is enriched near the transcription start site of genes affected by vitamin C treatment. Importantly, vitamin C, but not other antioxidants, enhances the activity of recombinant Tet1 in a biochemical assay, and the vitamin-C-induced changes in 5hmC and 5mC are entirely suppressed in *Tet1* and *Tet2* double knockout ES cells. Vitamin C has a stronger effect on regions that gain methylation in cultured ES cells compared to blastocysts, and *in vivo* are methylated only after implantation. In contrast, imprinted regions and intracisternal A particle retroelements, which are resistant to demethylation in the early embryo[2,13], are resistant to vitamin-C-induced DNA demethylation. Collectively, the results of this study establish vitamin C as a direct regulator of Tet activity and DNA methylation fidelity in ES cells.

ES cells are derived from the inner cell mass (ICM) of the blastocyst and can be cultured *in vitro* to maintain a pluripotent state. Media composition has been shown previously to influence ES-cell heterogeneity, gene expression and epigenetic patterns[14]. Our study began with the serendipitous observation that culture of mouse ES cells in knockout serum replacement (KSR) strongly and reversibly induces expression of the germline gene Dazl (Supplementary Fig. 1a, b). We performed a small-molecule screen and identified vitamin C as the KSR component responsible for Dazl induction (Supplementary Fig. 2). Dazl induction was also observed with the DNA methyltransferase (Dnmt) inhibitor 5-azacytidine, suggesting that vitamin C may promote DNA demethylation. Vitamin C enhances the activity of some Fe(II) 2-oxoglutarate dioxygenases[15], and we therefore reasoned that vitamin C could promote the activity of Tet enzymes, leading to DNA demethylation and Dazl induction. As mouse ES cells are commonly cultured without vitamin C, we set out to test the effect of vitamin C on the epigenetic and transcriptional state of ES cells.

Vitamin C treatment of naive ES cells cultured with MEK and GSK3β inhibitors (2i) in N2B27-based medium, which is devoid of detectable vitamin C (Supplementary Fig. 3), leads to a striking global increase in 5hmC by immunofluorescence and dot blot (Fig. 1a, b). In contrast, global levels of 5mC were not altered at 12 or 72 h after the start of vitamin C treatment (Fig. 1b). To assess dynamic 5hmC and 5mC changes at specific genomic regions, 5hmC and 5mC DNA immunoprecipitation followed by deep sequencing (DIP-seq) was performed at 12 and 72 h after vitamin C treatment. We restricted our analysis to methylated regions, as 5mC is a prerequisite for 5hmC.

Notably, most methylated promoters transiently gain 5hmC at 12 h and return to baseline levels or below at 72 h, whereas 5mC is lost progressively at 12 and 72 h (Fig. 1c, Supplementary Figs 4a and 5). Reduction of 5hmC at 72 h may be explained by loss of 5mC substrate. After 72 h of vitamin C treatment methylation is reduced by twofold or more in 61% of analysed promoters (Supplementary Table 1). Demethylation at exons, introns and intergenic regions is also observed (Supplementary Fig. 4b). There is a highly significant overlap in the promoters that gain 5hmC at 12 h and those that lose 5mC at 72 h ($P < 2.2 \times 10^{-16}$, Fig. 1d and Supplementary Fig. 6). In addition, 5hmC gain and 5mC loss occur at the same genomic locations near the transcription start site (TSS) (example in Fig. 1e). These results support a kinetic model in which oxidation of 5mC to 5hmC precedes DNA demethylation that may occur through active or passive mechanisms. We confirmed demethylation at the promoters of three representative genes by bisulphite sequencing (Fig. 1f). Several high-density CpG promoters show minimal demethylation and many of these were identified as imprinted genes (Fig. 1g), indicating that certain regions of the genome are resistant to vitamin-C-induced demethylation.

Although dot blot analysis indicates that the global rise in 5hmC is sustained at 72 h (Fig. 1b), DIP-seq indicates that promoters return to baseline 5hmC levels at this time (Fig. 1c). This apparent discrepancy may be explained by prolonged retention of 5hmC on repetitive elements, which cover a large portion of the genome. Indeed, we find that intracisternal A particle (IAP) endogenous retroviruses (ERVs) gain 5hmC at 12 h and maintain elevated levels after 72 h of vitamin C treatment (Fig. 1h). IAP retroelements are also resistant to vitamin-C-induced demethylation, as are other repetitive elements, which may explain the maintenance of 5mC observed in the dot blot (Fig. 1h, Supplementary Fig. 7a, b and Supplementary Table 2). The increase in 5hmC at IAP retroelements does not correspond to a loss in 5mC, which could occur if only a small fraction of methylated CpGs within these ERVs gain 5hmC, resulting in no detectable loss of overall methylation by DIP. Indeed, bisulphite sequencing reveals that IAP retroelements are not demethylated with vitamin C treatment at 72 h (Supplementary Fig. 7c).

The effects of vitamin C are specific, as several other antioxidants tested did not increase global 5hmC (Supplementary Fig. 8). The effects of vitamin C are also reversible. The global increase in 5hmC

[1]Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, Department of Obstetrics and Gynecology and Center for Reproductive Sciences, University of California San Francisco, 35 Medical Center Way, San Francisco, California 94143, USA. [2]Department of Medical Genetics, Life Sciences Institute, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada. [3]BC Cancer Agency, Canada's Michael Smith Genome Sciences Centre, 675 West 10th Avenue, Vancouver, British Columbia V5Z 1L3, Canada. [4]La Jolla Institute for Allergy and Immunology and Sanford Consortium for Regenerative Medicine, La Jolla, California 92037, USA. [5]Department of Microbiology and Immunology, Life Sciences Institute, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.

*These authors contributed equally to this work.

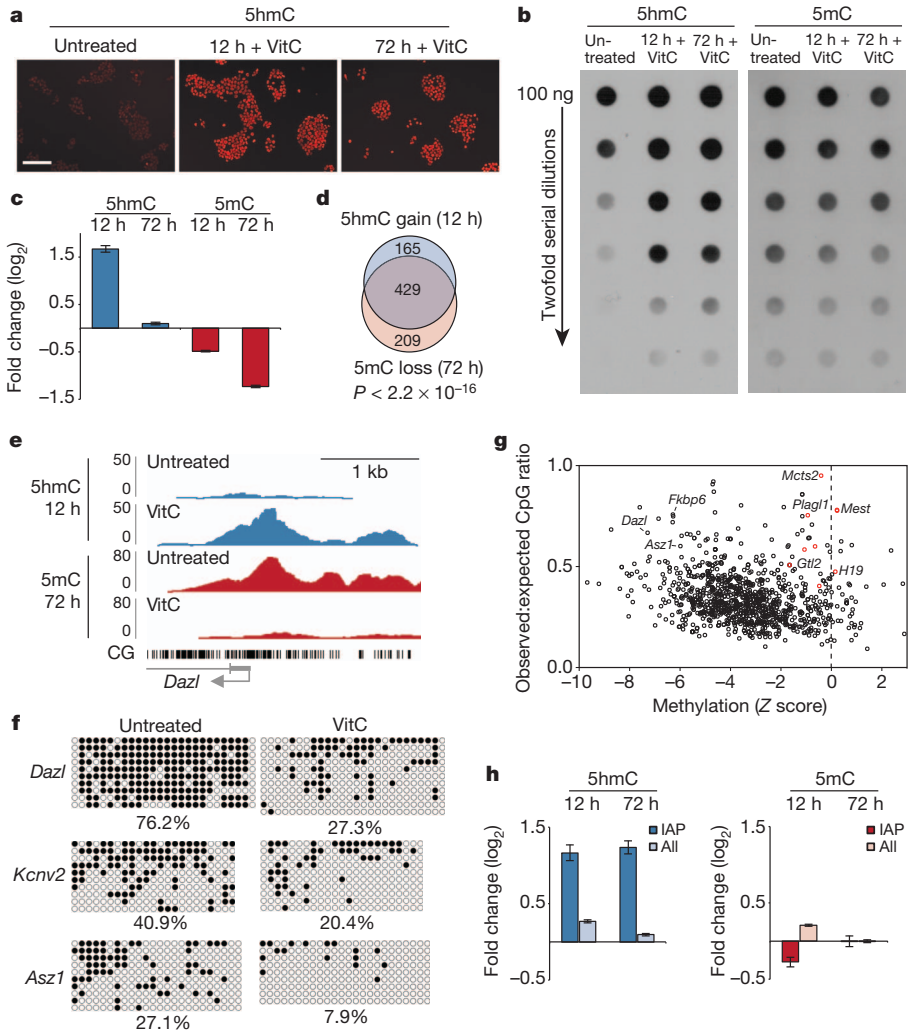

Figure 1 | Vitamin C induces loss of 5mC at gene promoters through a transient increase in 5hmC. **a**, Immunofluorescence for 5hmC. Scale bar, 200 μm. VitC, vitamin C. **b**, Global 5hmC and 5mC levels assayed by dot blot analysis. **c**, Graph shows fold change in DIP-seq reads (reads per kilobase per million, RPKM) at methylated promoters ($n = 1{,}045$) for vitamin-C-treated cells relative to untreated cells. Values are mean ± s.e.m. **d**, Overlap of methylated promoters that gain 5hmC and those that lose 5mC. *P* value was calculated using Fisher's exact test. **e**, Genome browser view of *Dazl*. **f**, Bisulphite sequencing of promoters. Open circles, unmethylated; closed circles, methylated. **g**, Scatter plot of methylated promoters comparing change in methylation (*Z* score) with CpG content. Red circles, imprinted genes. **h**, Graphs show fold change in RPKM at retrotransposons for vitamin-C-treated cells relative to untreated cells. Values are mean ± s.e.m.

is lost rapidly after 3 days of vitamin C withdrawal, whereas promoter 5mC increases gradually after vitamin C removal (Supplementary Fig. 9).

To determine how vitamin C affects gene expression in ES cells, microarray experiments were performed. Only approximately 200 genes are changed by more than twofold, and most are upregulated (Fig. 2a and Supplementary Table 3), consistent with loss of a silencing mark like 5mC. Upregulated genes are enriched on the X chromosome (32.7% observed versus 3.8% expected) and for germline gene ontology terms[16] (Fig. 2b, c). Pluripotency gene expression is not affected (Supplementary Table 3) and vitamin C treatment does not impair differentiation (Supplementary Fig. 10). Importantly, the expression of Tet and Dnmt genes is not affected by vitamin C treatment (Supplementary Fig. 11).

Germline genes are also induced in ES cells lacking Dnmts, as reported previously[17,18]. Out of the 134 vitamin-C-induced genes, 48 (36%) are also upregulated in *Dnmt1*$^{-/-}$;*Dnmt3a*$^{-/-}$;*Dnmt3b*$^{-/-}$ (*Dnmt* triple knockout) ES cells, which are devoid of DNA methylation (Supplementary Fig. 12a, b). Notably, vitamin C further increases expression of a subset of these genes in *Dnmt* triple knockout ES cells (Supplementary Fig. 12b), suggesting that vitamin C may regulate gene expression by additional mechanisms. For example, vitamin C may also stimulate histone demethylases, as has been shown in induced pluripotent stem cell (iPS cell) generation[19].

Genes upregulated by vitamin C have higher basal levels of promoter methylation in untreated cells (Fig. 2d). When analysis is restricted to germline genes (associated with the gene ontology term 'reproduction'), genes upregulated by vitamin C show even higher basal levels of promoter methylation (Fig. 2d). Furthermore, upregulated genes, particularly upregulated germline genes, show significant loss of methylation (Fig. 2e). Taken together, these results indicate that widespread promoter demethylation induced by vitamin C promotes the upregulation of predominantly germ line-associated genes. However, promoter demethylation is not sufficient to induce expression of most methylated genes, possibly owing to redundant epigenetic silencing mechanisms or a lack of activating transcription factors.

As Tet1, Tet2 and Tet3 are the only known enzymes that oxidize 5mC to 5hmC, we reasoned that the effects of vitamin C would be mediated by Tet enzymes. Indeed, vitamin C, but not other antioxidants such as glutathione or dithiothreitol (DTT), increases recombinant Tet1 activity in a dose-dependent manner in a biochemical assay (Fig. 3a). ES cells express two Tet family members, Tet1 and Tet2, and these enzymes seem to be highly redundant[20,21]. Tet1 binding[10] is enriched near the TSS of promoters that gain 5hmC or lose 5mC with vitamin C treatment (Supplementary Fig. 13). To test whether the effects of vitamin C are Tet-dependent, we analysed *Tet1*$^{-/-}$;*Tet2*$^{-/-}$

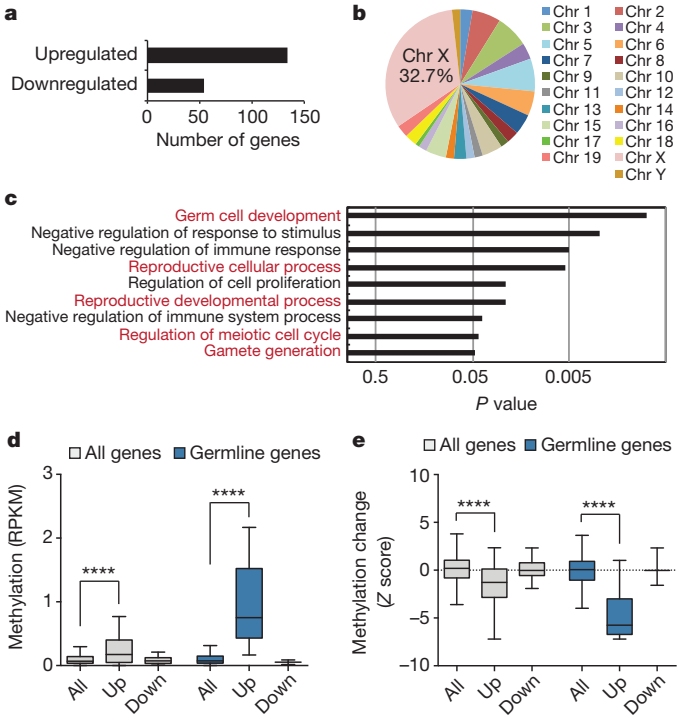

Figure 2 | Vitamin-C-induced DNA demethylation leads to expression of germline genes. **a**, Number of genes differentially expressed after vitamin C treatment (twofold and $P < 0.05$ by *t*-test). **b**, Chromosomal distribution of upregulated genes. **c**, Gene ontology analysis of upregulated genes. **d**, Box plot showing basal promoter methylation levels (RPKM) in untreated ES cells for all genes on the microarray ($n = 18{,}023$), upregulated genes ($n = 102$), downregulated genes ($n = 48$), all germline genes ($n = 865$), upregulated germline genes ($n = 8$), and downregulated germline genes ($n = 3$). **e**, Box plot showing the extent of vitamin-C-induced demethylation (*Z* score) at gene promoters categorized as in **d**. The box plots have Tukey whiskers, a line for the median, and edges for the 25th and 75th percentiles. ****$P < 0.0001$ by analysis of variance (ANOVA) throughout the figure.

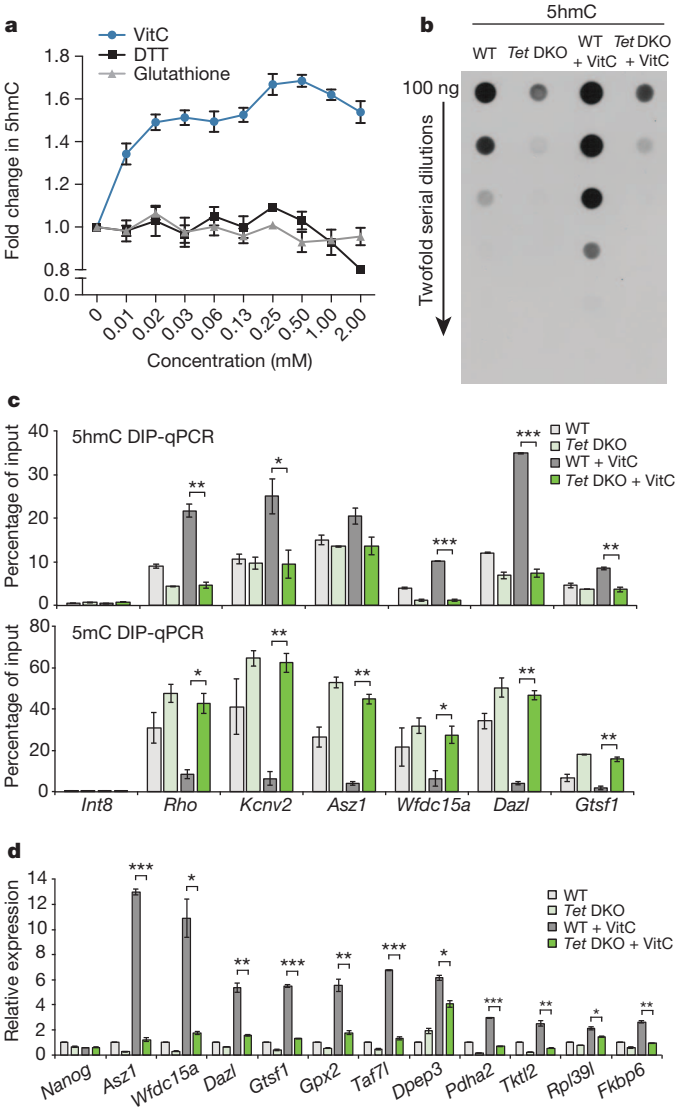

Figure 3 | The effects of vitamin C are Tet-dependent. **a**, Dose-dependent effect of vitamin C on *in vitro* Tet activity ($n = 3$ technical replicates, values are mean ± s.d.). **b**, Dot blot analysis for 5hmC after 12 h vitamin C treatment. DKO, double knockout; WT, wild-type. **c**, 5hmC (top) and 5mC (bottom) DIP-qPCR after 12 or 72 h vitamin C treatment, respectively. An intergenic region on chromosome 8 (Int8) is included as a negative control. **d**, Gene expression at 72 h of vitamin C treatment. Nanog, whose expression is not expected to change, is included as a control. qRT–PCR data expressed relative to untreated wild-type cells. For **c** and **d**, $n = 2$ biological replicates, values are mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by *t*-test throughout the figure.

(*Tet* double knockout) ES cells[21]. Dot blot analysis reveals that *Tet* double knockout ES cells show greatly reduced 5hmC signal that is not increased after vitamin C treatment (Fig. 3b). Residual signal in *Tet* double knockout ES cells may be due to antibody background or low-level Tet3 expression. Importantly, DIP followed by quantitative polymerase chain reaction (DIP-qPCR) reveals that in contrast to wild-type cells, vitamin C treatment of *Tet* double knockout ES cells does not affect 5hmC or 5mC levels at gene promoters (Fig. 3c). Furthermore, vitamin-C-induced gene expression is significantly attenuated in *Tet* double knockout ES cells (Fig. 3d). The modest gene induction observed may be due to effects of vitamin C unrelated to DNA methylation, as already suggested by the analysis of *Dnmt* triple knockout ES cells (Supplementary Fig. 12b). *Tet1*$^{-/-}$ (*Tet1* knockout) ES cells[20] also show an attenuated increase in global 5hmC, reduced promoter demethylation, and reduced gene induction in response to vitamin C. However, these effects are more subtle than in the *Tet* double knockout ES cells (Supplementary Fig. 14). These data indicate that the effects of vitamin C are Tet-dependent and are mediated by both Tet1 and Tet2.

Recent studies highlight differences in the methylomes of ES cells and blastocysts, with ES cells showing higher levels of methylation[13,22]. Using published genome-wide bisulphite sequencing data for ES cells and blastocysts[13], we investigated the relationship between vitamin-C-induced demethylation and differences in methylation between ES cells and the blastocyst. Analysis was performed on promoter CpG islands (CGIs) methylated in both our study and ref. 13 (Fig. 4a and Supplementary Table 4), which generally show greater methylation in ES cells than blastocysts (Fig. 4b). Interestingly, vitamin C induces greater demethylation at CGIs that are hypermethylated in ES cells relative to blastocysts (Fig. 4c, d). Conversely, vitamin C has modest effects on CGIs with similar methylation levels in ES cells and blastocysts, such as imprinted regions (Fig. 4c, d). IAP ERVs, which are similarly methylated in both ES cells and the blastocyst, are also resistant to vitamin-C-induced demethylation in ES cells (Supplementary Fig. 7d). These findings suggest that the effects of vitamin C are most pronounced at genes that show hypermethylation in ES cells compared to blastocysts.

Next, methylation dynamics during development were determined using a reduced representation bisulphite sequencing (RRBS) data set[2]. Gene promoters that show 5mC loss with vitamin C are generally unmethylated up to the ICM stage and then undergo extensive methylation at the epiblast stage (Fig. 4e, blue). In contrast, gene promoters that show 5mC maintenance with vitamin C are enriched for germ line differentially methylated regions (DMRs) that maintain approximately 50% methylation in somatic tissues throughout development

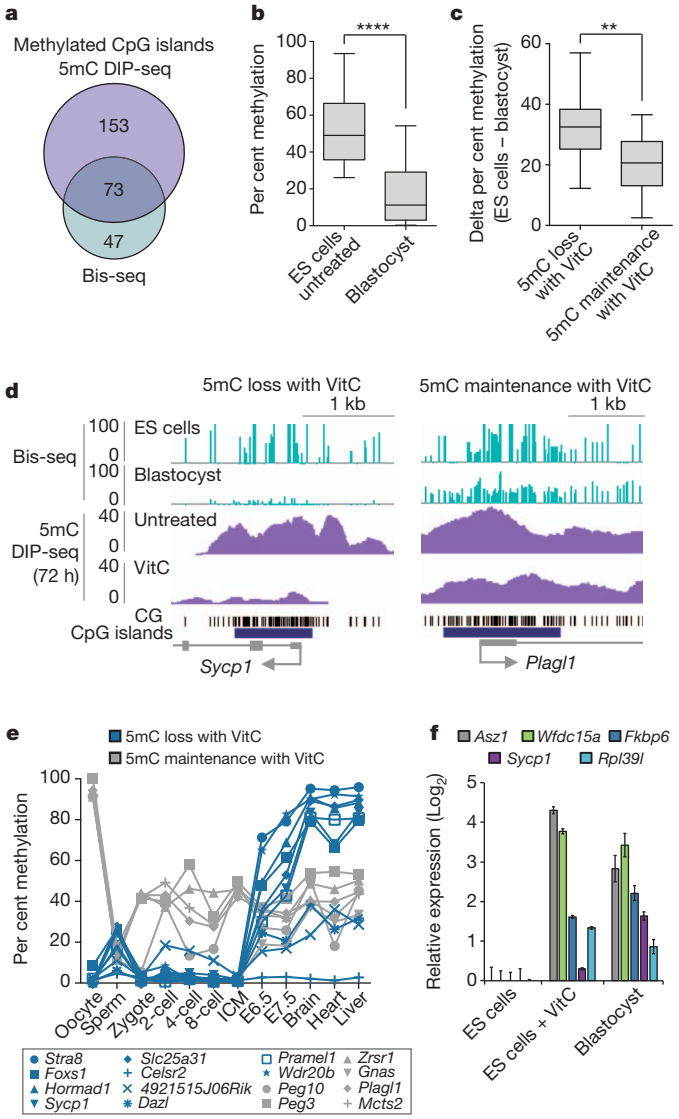

Figure 4 | Vitamin C reduces DNA methylation in ES cells that is normally gained post-implantation. **a**, Overlap of methylated CGIs in ES cells from this study (5mC DIP-seq, RPKM > 0.5) and ref. 13 (whole-genome bisulphite sequencing (Bis-seq), >25% methylated CpGs). Only CGIs found to be methylated in both data sets were used for subsequent analysis in **b**–**d**. **b**, The box plot shows CGI methylation levels in ES cells and blastocysts (values from ref. 13, $^{****}P < 0.0001$ by *t*-test). **c**, CGIs were first categorized as having 5mC loss with vitamin C (>75% loss of 5mC at 72 h, $n = 23$) or 5mC maintenance with vitamin C (<25% loss of 5mC at 72 h, $n = 14$) in ES cells, then plotted for difference in methylation between untreated ES cells and blastocysts. CGIs demethylated upon vitamin C treatment show significantly greater ES cell hypermethylation compared to CGIs resistant to vitamin C ($^{**}P < 0.01$ by *t*-test). **d**, Genome browser views of a gene from each category described in **c**. **e**, Methylation levels during development of all genes categorized in **c** for which data exist (see ref. 2). **f**, Gene expression in ES cells cultured with or without vitamin C compared to embryonic day 3.5 (E3.5) blastocysts. Data are expressed relative to untreated ES cells ($n = 2$ biological replicates, values are mean ± s.e.m.).

(Fig. 4e, grey). Thus, it seems that in ES cells cultured in the absence of vitamin C, methylation accumulates at the subset of CGIs that would normally be *de novo* methylated in the epiblast. Analysis of published RNA-seq data[23] indicates that several vitamin-C-induced genes are also expressed in the ICM (Supplementary Table 5). Furthermore, we find that several of these germline genes are indeed expressed in the blastocyst at levels comparable to ES cells treated with vitamin C (Fig. 4f). Collectively, these findings suggest that vitamin C remodels DNA methylation and expression patterns in ES cells, resulting in a state reminiscent of the ICM of the blastocyst.

Recently, it was reported that long-term culture of ES cells in 2i medium induces a blastocyst-like state of global hypomethylation relative to culture in serum[24]. As the analyses described above were carried out in 2i medium, they document effects of vitamin C beyond those of 2i. Nevertheless, we sought to distinguish the effects of 2i and vitamin C. We find that vitamin C induces a gain of 5hmC, loss of 5mC, and induction of germline genes in both FBS and 2i medium (Supplementary Fig. 15a–d). In contrast, culture in 2i medium alone shows little to no effect over the same 72-h time course. The faster kinetics of action of vitamin C relative to 2i are probably due to their different mechanisms of action: vitamin C promotes Tet-mediated DNA demethylation, whereas 2i promotes passive loss of DNA methylation via upregulation of Prdm14 and repression of Dnmt3b and Dnmt3l (ref. 24) (Supplementary Fig. 15e).

In summary, this work demonstrates that vitamin C alters the steady-state of DNA methylation and, in turn, the expression of germline genes in ES cells by enhancing Tet activity. Vitamin C reduces methylation at CGIs that normally gain methylation during the blastocyst to epiblast transition, promoting an ICM-like DNA methylation state in ES cells. Intriguingly, although human ES cells are normally cultured in medium containing vitamin C, they resemble mouse epiblast cells more than ICM cells. Nevertheless, vitamin C may also have a role in human ES cells, as it has been shown that they accumulate DNA methylation after several passages in the absence of vitamin C, although the underlying mechanisms were not addressed[25]. Notably, imprinted regions and IAP retroelements, which are resistant to DNA demethylation in the early embryo[2,13], are also resistant to vitamin-C-mediated demethylation. These regions show high levels of H3K9me3 (ref. 18), suggesting that this mark, or readers of this mark, may have a role in protecting against Tet-mediated demethylation. Vitamin C also improves the quality of iPS cells by preserving the fidelity of DNA methylation at imprinted regions[26]. Furthermore, Tets are required for methylation reprogramming during iPS cell generation and in the zygote[4,27,28]. Vitamin C has also been reported to increase 5hmC in mouse embryonic fibroblasts[29], suggesting that the mechanism characterized here may be broadly applicable to other cell types. Much work remains to be carried out to evaluate the ability of vitamin C to modulate Tet activity and DNA methylation *in vivo*. It will be of interest to investigate the role of vitamin C in contexts in which Tet enzymes have been implicated[30], such as in the zygote, germ line, blood and brain. Potential roles for vitamin C in the clinic, including in *in vitro* fertilization culture medium or in cancers driven by aberrant DNA methylation, also deserve exploration.

METHODS SUMMARY

ES cells were cultured in feeder-free conditions in 2i medium. Vitamin C (L-ascorbic acid 2-phosphate, Sigma) was added daily at 100 μg ml^{-1}. The Tet activity assay was performed using recombinant human Tet1 catalytic domain with 5hmC generation quantified by enzyme-linked immunosorbent assay (ELISA). Dot blot analyses were performed with serial dilutions of DNA using a Bio-Dot (Bio-Rad) apparatus and antibodies against 5hmC or 5mC. For gene expression analysis, total RNA was isolated and hybridized to Affymetrix mouse gene 1.0 ST GeneChip arrays or analysed by qPCR with reverse transcription (qRT–PCR). For 5mC DIP-seq and 5hmC DIP-seq, immunoprecipitated DNA was adaptor-ligated for paired-end sequencing on an Illumina HiSeq following the manufacturer's recommended protocol. Sequence reads were aligned to the mm9 mouse reference genome and unique reads were used to calculate RPKM values in various regions including RefSeq promoters and CGIs. For pair-wise sample comparisons, an empirical *Z* score was calculated assuming the distribution of RPKMs for each sample followed a Poisson model.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.R.-S. directed the study. K.B. and K.T.E. designed and performed experiments. K.B., K.T.E., M.M.K., M.C.L. and M.R.-S. analysed and interpreted the data. M.M.K. and M.C.L. performed bioinformatics analyses. P.G. performed bisulphite sequencing. S.M. generated the human recombinant Tet1 catalytic domain, and J.A.Z.-M. performed the *in vitro* Tet activity assay under the direction of A.R., who provided expertise on Tet activity. A.T. and M.H. developed and performed DIP-seq and sequencing data processing. D.J.L. provided financial support, advice, and laboratory space to K.T.E. K.B., K.T.E., M.C.L. and M.R.-S. wrote the manuscript.

Author Information Microarray and DIP-seq data have been deposited in the Gene Expression Omnibus under the accession number GSE46403. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.C.L. (mlorincz@mail.ubc.ca) or M.R.-S. (mrsantos@diabetes.ucsf.edu).

METHODS

Cell culture. ES cells used in this study include: *Oct4*-GiP (129 × MF1), V6.5 (129/Sv × C57/BL6), *Tet1*$^{-/-}$, *Tet1*$^{-/-}$ *Tet2*$^{-/-}$, J1 (129/Sv), and *Dnmt1*$^{-/-}$ *Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$. All ES cells were cultured in feeder-free conditions on 0.1% gelatin-coated tissue culture plates. Unless indicated otherwise, ES cells were cultured in 2i medium, which is composed of a N2B27 base medium[31] supplemented with the MEK (MAP-kinase kinase) inhibitor, PD0325901 (1 μM, Stemgent), the GSK3β inhibitor, CHIR99021 (3 μM, Selleck Chemicals), and with ESGRO leukaemia inhibitory factor (LIF) at 1,000 units per ml (Millipore). Vitamin C (L-ascorbic acid 2-phosphate, Sigma, A8960) was added on day 1 after seeding at 100 μg ml^{-1}. Medium was replaced daily. For KSR versus FBS studies, a base medium composed of high glucose DMEM (Life Technologies), L-glutamine (2 mM, Life Technologies), sodium pyruvate (1 mM, Life Technologies), non-essential amino acids (1×, Life Technologies), 2-mercaptoethanol (1×, Millipore), and penicillin-streptomycin (1×, Life Technologies) was supplemented with LIF (1,000 units per ml) and either 15% KSR (KSR medium) or 15% FBS (FBS medium). For differentiation experiments, *Oct4*-GiP ES cells maintained in 2i medium were treated with vitamin C for 72 h. Untreated and vitamin-C-treated ES cells were then transferred to 60-mm Petri dishes and cultured in suspension in FBS medium without LIF to induce embryoid body formation. Medium was replaced on day 3 and embryoid bodies were collected on day 5 of differentiation. To compare the effects of vitamin C in FBS medium versus 2i medium, J1 ES cells maintained in FBS medium were switched to FBS medium plus vitamin C, 2i medium, or 2i medium plus vitamin C and collected at 12 and 72 h after changing conditions. Media were replaced daily.

Small molecule screen. *Oct4*-GiP ES cells were cultured in FBS medium. Small molecules were added at the time of seeding. The small molecules used included valproic acid (2 mM, Calbiochem), trichostatin A (20 nM, Sigma), PD0325901 (1 μM, Stemgent), CHIR99021 (3 μM, Selleck Chemicals), forskolin (10 μM, Sigma), A-83-01 (1 μM, Stemgent), RG108 (250 nM, Stemgent), 5-azacytidine (1 μM, Sigma), BIX01294 (0.5 μM, Stemgent), UNC0638 (0.5 μM, Sigma), insulin (5 μg ml^{-1}, Sigma), vitamin C (100 μg ml^{-1}, Sigma) and the lipid cocktail Albumax II (5 mg ml^{-1}, Life Technologies). The KSR components tested include vitamin C, Albumax II, and insulin (international patent application WO 98/30679).

Vitamin C quantification assay. The amount of vitamin C in 2i medium was determined using an Ascorbic Acid Assay Kit (Abcam, ab65656). Known amounts of vitamin C (L-ascorbic acid, Sigma, A4544) were added to the medium and tested as controls.

Antioxidant treatment. *Oct4*-GiP ES cells were cultured in 2i medium and treated for 24 h with antioxidants. The antioxidants tested included a modified glutathione, glutathione reduced ethyl ester (GMEE), at 1.5 μg ml^{-1}, sodium selenite (20 nM), vitamin B1 (9 μg ml^{-1}), vitamin E (25 μM), L-carnitine hydrochloride (15 μg ml^{-1}), and α-lipoic acid (5 μg ml^{-1}). All reagents were purchased from Sigma. After treatment, DNA was isolated and assayed by dot blot analysis.

Immunofluorescence staining. Cells were fixed in 4% paraformaldehyde for 15 min. After washing three times with PBS, cells were blocked with 5% FBS in PBST (PBS + 0.5% Tween 20) for 2 h at room temperature (20–25 °C). Primary antibodies were diluted in blocking solution and incubated with cells overnight at 4 °C. Cells were then washed three times in PBS and incubated for 2 h at room temperature with secondary antibodies diluted in blocking solution. Cells were washed three times in PBS before imaging. Primary antibodies included DAZL (1:200, Abcam) and 5-hydroxymethylcytosine (1:100, Active Motif). 594-conjugated chicken anti-rabbit (1:1,000, Life Technologies) was used as a secondary antibody.

Genomic DNA preparation. DNA was isolated using a Qiagen Gentra Puregene Kit or the phenol-chloroform-isoamyl alcohol method. RNase A digestion was included in the isolation procedure.

Dot blot analysis. Isolated DNA (1 μg per sample) was denatured in 0.1 M NaOH for 10 min at 95 °C. Samples were neutralized with 1 M NH_4OAc on ice, and then serially diluted twofold. DNA samples were spotted on a nitrocellulose membrane using a Bio-Dot apparatus (Bio-Rad). The blotted membrane was washed in 2× SSC buffer, dried at 80 °C for 5 min, and UV cross-linked at 120,000 μJ cm^{-2}. The membrane was then blocked in Odyssey buffer (Li-Cor) diluted 1:1 in PBS (Odyssey:PBS) overnight at 4 °C. Mouse anti-5-methylcytosine monoclonal antibody (Active Motif, 1:500) or rabbit anti-5-hydroxymethylcytosine polyclonal antibody (Active Motif, 1:5,000) in Odyssey:PBS was added for 3 h at room temperature. The membrane was washed for 10 min three times in PBS, and then incubated with either HRP-conjugated sheep anti-mouse immunoglobulin-G (IgG) (GE, 1:10,000) or HRP-conjugated goat anti-Rabbit IgG (Abcam, 1:10,000) secondary antibodies in Odyssey:PBS for 3 h at room temperature. The membrane was then washed for 10 min three times in PBS and visualized by chemiluminescence with GE ECL Plus.

qRT–PCR. Total RNA was isolated from cultured cells using Qiagen RNeasy with on-column DNase I treatment. Complementary DNA (cDNA) was generated from 1 μg of RNA using random hexamers to prime the reaction. The cDNA was used as template for qRT–PCR. qRT–PCR was performed in combination with the KAPA SYBR Fast ABI Prism qPCR kit on an Applied BioSystems 7900HT sequence detection system. Primer sequences are listed in Supplementary Table 6. The relative amount of each gene was normalized using two housekeeping genes (*L7* (also known as *Rpl7*) and *Ubb*), unless otherwise specified.

Blastocyst expression analysis. C57BL/6 (Simonsen) female mice were injected with 7.5 IU of pregnant mare gonadotropin (HUMC-NHPP) followed by 7.5 IU human chorionic gonadotropin (Sigma) 46 h later. Primed females were mated with DBA/2 (Simonsen) male mice. Detection of a vaginal plug was defined as 0.5 days post coitum. Embryonic day 3.5 blastocysts were obtained by flushing the uterus of superovulated females with M2 medium (Sigma). Total RNA was isolated from collected blastocyst using a RNeasy Micro Kit (Qiagen) with on-column DNase I treatment. qRT–PCR was performed as described above. The relative amount of each gene was normalized to *L7*. All animal work was conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Microarray analysis. Total RNA was isolated from biological triplicates of *Oct4*-GiP ES cells treated with or without vitamin C for 72 h and hybridized to Affymetrix mouse gene 1.0 ST GeneChip arrays (Affymetrix). Only annotated probes were considered for analysis. DChip software was used for gene expression statistical analysis.

Gene ontology analysis. Gene ontology functional annotation was performed in DAVID.

***In vitro* recombinant Tet1 activity assay.** Recombinant human TET1 catalytic domain (1.2 μg) was incubated for 10 min at 37 °C with 178.2 ng of biotinylated DNA substrate in 50 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM α-ketoglutarate, 3.7 μM ammonium iron (II) sulphate hexahydrate, 0.1 mg ml^{-1} BSA, 1 mM ATP and titrating concentrations of vitamin C (Sigma, A5960), DTT (Sigma, D0632) or reduced glutathione (Sigma, G6529). The reaction was quenched by adding EDTA (11 mM). Each sample was loaded into a 384-well streptavidin coated plate in triplicates (25-μl well) and left overnight at 4 °C on a rotating platform. The next morning the wells were washed three times with 90 μl of 1× Tris-buffered saline (TBS) with 0.1% Tween (TBST). The wells were then incubated with 50 μl per well of anti-5hmC antibody (Active Motif) diluted 1:5,000 in TBST with 5% milk for 1 h at room temperature on a rotating platform. Next the wells were washed three times with 90 μl of TBST, followed by incubation with 50 μl of goat anti-rabbit peroxidase diluted 1:3,000 in TBST with 5% milk for 1 h on a rotating platform. The wells were then washed three times with TBST, and 30 μl of TMB substrate reagent mix (BD Biosciences) were added to each well. The reaction proceeded in the dark for 10 to 12 min, and was then quenched with 20 μl of 25% sulphuric acid. The absorbance of each well was measured at 450 nm. A standard curve of twofold dilutions from a fully hydroxymethylated version of the DNA substrate was used to obtain a linear regression, which was used to calculate pmols of 5hmC formed in the reaction from absorbance values. The data are presented as fold change in 5hmC relative to untreated.

Bisulphite Sanger sequencing. *Oct4*-GiP ES cells treated with or without vitamin C for 72 h were analysed for promoter methylation. To analyse the methylation status of the selected genes, 0.2 μg of genomic DNA was subject to sodium bisulphite conversion using the EZ DNA Methylation-Gold kit (Zymo Research). Primers specific for the genes analysed (Supplementary Table 6) were employed in nested or semi-nested PCR reactions. PCR products were cloned through TA cloning using the pGEM-T easy kit (Promega) and individual inserts were sequenced using Genewiz Sequencing. Data were analysed using Quantification Tool for Methylation Analysis[32]. The percentage of methylated CpGs sequenced is presented for each set of samples.

5mC and 5hmC DIP-qPCR. DIP was performed using the Diagenode MagMeDIP or hMeDIP Kit with minor modifications. DNA was sonicated into short fragments (100 to 1,000 base pairs (bp)) with a Diagenode Bioruptor for 20 min with 15-s on, 15-s off cycles at low power. Sonicated DNA was heat denatured at 95 °C for 10 min. Sonicated DNA (1 μg) was immunoprecipitated with 1 μg of mouse anti-5-methylcytosine monoclonal antibody (Active Motif, 1 μg μl^{-1}) or 2.5 μg of mouse anti-5-hydroxymethylcytosine monoclonal antibody (Diagenode, 1 μg μl^{-1}). After a 2-h incubation at 4 °C, Magbeads (Diagenode) were added to the DNA-antibody mixture and samples were incubated at 4 °C overnight. Isolation of immunoprecipitated DNA was performed according to the kit instructions. qPCR was performed in combination with the KAPA SYBR Fast ABI Prism qPCR kit on a Applied BioSystems 7900HT Sequence Detection System. Primer sequences are listed in Supplementary Table 6.

5hmC and 5mC DIP-seq. *Oct4*-GiP ES cells were treated with vitamin C (100 μg ml^{-1}) for 12 and 72 h. For each sample, 12 μg of genomic DNA was isolated, split into three replicates of 4 μg each and sonicated to approximately 100 to 500 bp on a Covaris E210 platform (75 s, 10% duty cycle). Sheared DNA was end-repaired,

A-tailed, and ligated to custom paired-end adapters as described[33]. Ligated genomic DNA was size selected (100 to 300 bp) by 8% PAGE (Nuvex, Invitrogen) to remove unligated adapters. Replicates were pooled and subjected to qPCR using truncated PE1.0/2.0 PCR primers to assess ligation efficiency and quantified by fluorometer (Qubit, Life Technologies). Adaptor-ligated DNA was heat denatured at 95 °C for 10 min, rapidly cooled on ice, and immunoprecipitated with a mouse monoclonal anti-methylcytidine antibody (1 mg ml^{-1}, Eurogentec) or a mouse monoclonal anti-5-hydroxymethylcytidine (5hmC) antibody (1.6 mg ml^{-1}, Diagenode). Primary antibodies were added at 1 µl µg^{-1} of DNA and samples were incubated overnight at 4 °C with rocking agitation in 500 µl IP buffer (10 mM sodium phosphate buffer, pH 7.0, 140 mM NaCl, 0.05% Triton X-100). To recover the immunoabsorbed DNA fragments, 1 µl of rabbit anti-mouse IgG secondary antibody (2.5 mg ml^{-1}, Jackson ImmunoResearch) and 100 µl Protein A/G beads (Pierce Biotechnology) were added and incubated for 2 h at 4 °C with agitation. After immunoprecipitation beads were resuspended in TE buffer with 0.25% SDS and 0.25 mg ml^{-1} proteinase K for 2 h at 55 °C and then allowed to cool to room temperature. Immunoprecipitated and supernatant DNA were purified using Qiagen MinElute columns and eluted in 16 µl EB (Qiagen). Sequencing libraries were generated by 10 to 15 cycles of PCR using custom indexed paired-end Illumina PCR primers[34]. The resulting reactions were purified over Qiagen MinElute columns, after which a final size selection was performed by electrophoresis in 8% PAGE. Libraries were quality controlled by spectrophotometry and Agilent DNA Bioanalyzer analysis. An aliquot of each indexed library was pooled by sample and sequenced two per lane on an Illumina HiSeq2000 platform (2X76 nt + 7 nt) following the manufacturer's recommended protocol and V3 chemistry (Illumina).

Bioinformatics. Sequence reads (75 bp paired-end) were aligned to the mouse reference genome (mm9) using BWA v0.5.9 (ref. 35) and default parameters. Samtools[36] and Picard (http://picard.sourceforge.net/) were used to sort and mark duplicate reads respectively. Reads having identical coordinates were collapsed into a single read and reads with mapping qualities ≥ 5 passed to FindPeaks 4.01 (ref. 37) for generation of unthresholded and thresholded (false discovery rate (FDR) < 0.01) coverage wig files to be visualized in the UCSC genome browser[38]. To quantify the strength of 5hmC and 5mC DIP-seq marks, these wig files were used to calculate reads per kilobase per million reads (RPKM)[39,40] values in various regions of interest including RefSeq[41] promoters and CGIs (downloaded from http://genome.ucsc.edu on May 15, 2012). For pair-wise sample comparisons, an empirical Z score was calculated assuming the distribution of RPKMs for each sample followed a Poisson model: Z score $= (RPKM_A - RPKM_B)/\sqrt{(RPKM_A - r_{AB}RPKM_B)}$, where $RPKM_A$ and $RPKM_B$ are RPKMs in the region of interest of A and B samples, respectively, and $r_{AB} = N_A/N_B$, where N_X is the total number of aligned reads used for normalization. For promoter analysis, promoters were defined as ±500 bp from the TSS. Methylated promoters were defined as having >0.5 RPKM values in both 12 and 72 h untreated 5mC DIP-seq samples ($n = 1{,}045$). For previously published Tet1 ChIP-seq (chromatin immunoprecipitation followed by sequencing) data (ref. 10), short read data were downloaded from the NCBI GEO archive (GSE24843) and remapped to mm9 (NCBI 37). ChIP-seq reads with identical coordinates were collapsed into a single read. Tet1 reads were directionally extended by 300 bp using FindPeaks and unthresholded coverage wig files were generated to create Tet heatmaps in methylated promoters using ChAsE[42], an interactive analysis and exploration tool for epigenetic data. Tet1 ChIP-seq data was corrected by control data. To determine which retroelement subfamilies show global 5hmC and 5mC DIP-seq changes in vitamin-C-treated compared to untreated ES cells, RPKM values were calculated for all subfamilies in each 5hmC and 5mC DIP-seq data set. To generate RPKM values, we calculated the total number of reads aligned to each retroelement subfamily using both unique and multiple-aligned reads, and normalized the total number of reads by total genomic bp per subfamily and total number of reads for each data set. For whole genome bisulphite-seq analysis of CGIs, the percentage of methylation for each individual cytosine was downloaded from ref. 13 (http://www.nodai-genome.org/mouse_en.html). CGI methylation levels were calculated as the average of the percentage of methylation for all cytosines in each CGI region. Analysis was restricted to CGIs ± 1,000 bp from a TSS.

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